# A STUDY OF TWO DISTINCT STRAINS OF STREPTOCOCCUS ISOLATED FROM THE SAME HEART-VALVE LESION

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Studies which have had for their aim the classification of the streptococcus group are numerous. While they have led to important additions to our knowledge of the group, they have left many questions relating to classification and to biologic reactions and variations still in doubt. The cultural studies of Andrewes and Horder,[1] Holman,[2] and Brown [3] have simplified the classification and permit ready grouping by reactions on sugar and blood mediums, but they have disregarded the immunologic relations; strains of streptococci which are culturally distinct may be immunologically identical. The application of immunologic reactions alone has yielded no more satisfactory results. In our own studies of complement-fixation reactions,[4] there was no correlation between complement fixation and the reactions on blood mediums, nor between complement fixation and grouping based on fermentative reactions; neither was there any distinct correlation between complement fixation and disease grouping. Dochez, Avery and Lancefield [5] obtained evidence of grouping by agglutination reactions. Their work, however, was limited to hemolytic strains; the results with complement fixation suggest that it is probable that some nonhemolytic strains, differing from those studied in so fundamental a property as the reaction on hemoglobin, will fall into the groups based on agglutination. The relative importance of environmental factors during the stage of parasitism, i. e., host species, tissue localization and pathologic process, and of cultural reactions during the stage of cultivation outside the body has not been sufficiently investigated.

The isolation of two morphologically and culturally distinct species of streptococci from the same lesion so deep seated as to exclude the fortuitous addition of one to the other from the surface offered an

Received for publication, Oct. 28, 1921.

1 Lancet, 1906, 2, pp. 708, 775, 852.
2 Jour. Med. Research, 1916, 24, p. 377.
3 Monographs of the Rockefeller Institute for Medical Research, No. 9, 1919.
4 Jour. Infect. Dis., 1918, 28, p. 230.
5 Jour. Exper. Med., 1919, 30, p. 179.

opportunity to study some of these questions, and, more particularly, to determine whether the two invading organisms might have identical reactions as the result of growth in the same host, the same tissue and the same lesion. Furthermore, it appeared important to determine the fixity of the reactions by repeated investigation of the same two organisms during a prolonged period of cultivation outside the body, since several recent writers have reported the development of nonhemolytic colonies from hemolytic strains and of hemolytic variants from nonhemolytic strains.

The two strains were isolated from a vegetative growth on the aortic valve, obtained at necropsy Oct. 4, 1919. Clinically the case was a typical chronic infectious endocarditis. The lesion from which the organisms were isolated was a chronic vegetative aortic valvular endocarditis. The first cultures were obtained by grinding the vegetative growth with sterile sand, in a sterile mortar, and by streaking the fluid material thus obtained on the surface of blood agar plates. There was no growth on the blood-agar plates in 12 hours, but in 36 hours there were a number of small, round, elevated, white, moist colonies which were surrounded by a clear zone of hemolysis, 2 to 3 mm. in diameter. In 48 hours there were, in addition to the hemolytic colonies, minute, moist, elevated, green colonies, with no surrounding zone of hemolysis. The blood-agar plates were examined daily, and after 5 days, the hemolytic colonies had undergone no change, but the green colonies had turned to a brownish color.

Films made from a hemolytic colony and stained by the Gram method, showed a small, round, gram-positive coccus, in pairs and in chains of varying length (fig. 1). Films from a green colony, and similarly stained, showed a more minute gram-positive diplococcus, usually forming chains (fig. 2). The difference in the size of the two cocci is even more striking on direct visual examination than in the photomicrographs.

Which of the two streptococci was the primary cause of the heart lesion it is impossible to determine definitely. The aortic lesion was of the type usually associated with Streptococcus viridans. In the pulmonary artery, just at the bifurcation, there was a large, raised soft, warty vegetation, which on both gross and microscopic examination was more recent than the aortic lesion. From the pulmonary artery the green streptococcus alone was isolated. This would seem to indicate that the latter organism had the greater invasive powers; however, virulence tests made immediately after isolation of the two

strains showed the hemolytic organism to be much more highly virulent for laboratory animals than the viridans strain. The absence of more acute ulcerative reaction in the aortic valve, such as is usually

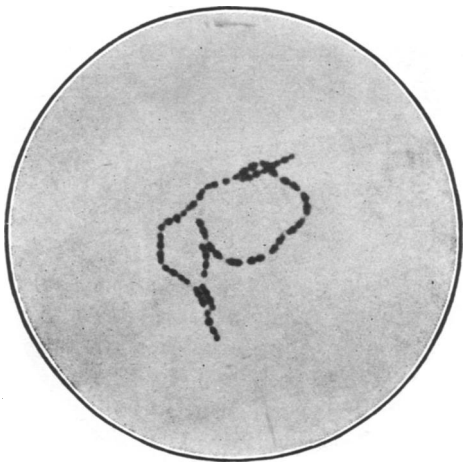

Fig. 1.—Hemolytic streptococcus; Gram stain; × 1200.

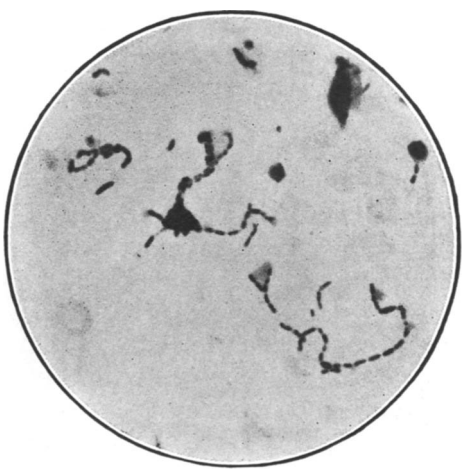

Fig. 2.—Viridans streptococcus; Gram stain; × 1200.

associated with the hemolytic streptococcus, in spite of the greater virulence of the latter, may be interpreted as evidence that this organism was a later invader of a lesion primarily due to Streptococcus viridans.

The two strains of streptococcus were subcultivated in ascites meat-infusion dextrose broth of a $P_H$ 7.6 reaction. After 24 hours, the broth tube, inoculated with the hemolytic streptococcus, had a flocculent sediment and a cloudy supernatant; the broth tube inoculated with the nonhemolytic streptococcus had a granular sediment and a clear supernatant fluid. Neither coccus was soluble in bile nor did either ferment inulin. Classified on a basis of sugar fermentations (Andrewes and Horder,[1] or Holman [2]), the hemolytic streptococcus belonged to the Streptococcus pyogenes group, and the nonhemolytic streptococcus belonged to the streptococcus salivarius group. Neither streptococcus was a mannitol fermenter.

The virulence of the two strains was tested by intraperitoneal injection of white mice with varying doses of 18-hour growths of the bacteria in broth medium. The lethal dose of the hemolytic streptococcus was 0.06 c c of the 18-hour broth culture. The viridans streptococcus had little pathogenicity for white mice, since a whole c c of an 18-hour broth culture (having approximately the same number of organisms as did the corresponding hemolytic streptococcus) failed to kill a white mouse, although the latter was apparently quite ill for several days. Mice receiving smaller doses of this nonhemolytic streptococcus, were apparently not affected. The virulence tests were repeated after 6 weeks. The viridans streptococcus, as far as could be detected, was entirely nonpathogenic for white mice at that time. The virulent quality of the hemolytic streptococcus was almost entirely lost, since it required 1.5 c c of the 18-hour broth culture to kill a mouse. The virulence of the hemolytic streptococcus was not appreciably increased by repeated animal passage. As the hemolytic streptococcus lost its virulence, it also lost the peculiar quality, previously recorded,[6] of agglutinating instead of laking red blood cells of various species, when it was grown in broth medium.

During the two years in which the strains of streptococcus have been under observation, half-grown rabbits were immunized at different times, and agglutination, opsonic, and complement-fixation tests were made with immune serums. After a number of preliminary tests, it was decided that serum, inactivated by heating at 56 C. for 30 minutes, was more satisfactory for use in the tests than active serum, since it was less apt to be anticomplementary in the fixation tests, and at the same time it was equally good for the agglutinin and opsonin tests. Some difficulty was experienced in obtaining smooth suspensions,

[6] Jour. Infect. Dis., 1920, 27, p. 565.

since both strains were inclined to clump spontaneously in broth medium, but by transferring daily, from one ascites meat-infusion dextrose broth to another, a comparatively smooth suspension resulted. The 18-hour growths were centrifugated, the supernatant broth removed, the precipitated bacteria washed with salt solution, and resuspended in salt solution in approximately the same concentration as that of the 18-hour broth culture, shaken with sterile glass beads, centrifugated a few minutes at low speed to remove any small clumps, and the fluid removed and heated at 56 C. for 30 minutes.

*Agglutination.*—The macroscopic method was used. To varying dilutions of a normal rabbit serum and to varying dilutions of each immune serum was added an equal amount of killed bacterial suspension. They were mixed in tubes, and the tubes were placed in the hot water bath at 56 C. for one hour. The tests were read at once and the highest dilution of serum that agglutinated the bacteria was noted and is indicated by the figures recorded in the table. Whenever there was any clumping in the control tube that contained bacterial suspension only, or in any of the tubes containing normal serum and suspension, the tests were discarded.

*Opsonic Tests.*—Opsonin was estimated by the opsonic index method, and the figures are recorded in the table.

*Complement Fixation.*—The tests were made according to the original Wassermann test (one-tenth method). Hot water incubation was used, and the readings were made and recorded immediately after the second incubation. A normal rabbit control serum was set up with each test, and unless there was complete hemolysis with this serum, the tests were discarded. When there was complete inhibition of hemolysis with $\frac{1}{4}$ and $\frac{1}{8}$ of the anticomplementary unit of antigen, the test was considered weakly positive, and is indicated in the table by $+$. Fixation with $\frac{1}{16}$ and $\frac{1}{32}$ is indicated by $++$, fixation with $\frac{1}{64}$ and $\frac{1}{128}$ by $+++$, and fixation with $\frac{1}{256}$ or over, by $++++$

Three rabbits were immunized with the hemolytic streptococcus strain and 3 rabbits with the nonhemolytic streptococcus strain during the 2 years in which the strains were under observation. Frequent tests were made on the rabbit serums during the immunization period, but the observations recorded in the table are those in which each immune serum had the maximum reaction with the homologous suspension in the tests.

Two rabbits were immunized in 1919 with the freshly isolated strains. The hemolytic streptococcus immune serum (hemolytic streptococcus serum 1) agglutinated the hemolytic streptococcus in 1:640 serum dilution, and the nonhemolytic streptococcus in 1:80; the opsonic index to hemolytic streptococcus

was 2.5, and to the nonhemolytic streptococcus 3.5; in the complement-fixation test, the immune serum gave + + + fixation with the homologous antigen, and no fixation with the heterologous antigen. The nonhemolytic immune serum (nonhemolytic streptococcus serum 1) agglutinated the nonhemolytic streptococcus suspension in 1:2560 serum dilution, but it did not agglutinate the hemolytic streptococcus suspension at all; the opsonic index to the nonhemolytic streptococcus was 3.5 and to the hemolytic streptococcus 3; in spite of the high agglutinating titer of this serum, the complement-fixation antibodies were low, + with the nonhemolytic streptococcus antigen, and negative with the hemolytic streptococcus antigen.

TABLE 1

TABLE OF SEROLOGIC REACTIONS

| Immune Serums | Agglutinin | | Opsonic Index | | Complement Fixation | |
|---|---|---|---|---|---|---|
| Hemolytic Streptococcus | Hemolytic Streptococcus Suspension | Nonhemolytic Streptococcus Suspension | Hemolytic Streptococcus Suspension | Nonhemolytic Streptococcus Suspension | Hemolytic Streptococcus Antigen | Nonhemolytic Streptococcus Antigen |
| 1 | 640 | 80 | 2.5 | 3.5 | +++ | 0 |
| 2 | 40 | 20 | 1.5 | 1.0 | + | + |
| 2A | 1.280 | 160 | 4.0 | 1.0 | ++++ | + |
| 3 | 640 | 160 | 3.2 | 2.0 | ++ | 0 |
| 3A | 1,280 | 1,280 | ... | ... | ++++* | ++++* |
| Nonhemolytic Streptococcus | | | | | | |
| 1 | 0 | 2,560 | 3.0 | 3.5 | 0 | ++ |
| 2 | 10 | 2,560 | 4.0 | 2.0 | + | ++++ |
| 2A | 160 | 2,560 | 4.0 | 2.0 | + | ++ |
| 3 | 40 | 640 | 2.0 | 1.0 | 0 | + |
| 3A | 1,280 | 1,280 | ... | ... | ++++* | ++++* |

* Anticomplementary.

Two more rabbits were immunized during the spring of 1920. The hemolytic streptococcus serum was very low in antibody content; since it was impossible to raise the titer at this time, the rabbit was bled. The results of the tests with this serum (hemolytic streptococcus serum 2) were as follows: agglutination with hemolytic streptococcus suspension, 1:40, with nonhemolytic streptococcus suspension 1:20; opsonic index to hemolytic streptococcus, 1.5 and to nonhemolytic streptococcus, 1; complement-fixation was + with both antigens. It was thought that the antibody producing organs might have been overstimulated, and the rabbit was permitted to rest for about 3 months, and then it again received immunizing doses of hemolytic streptococci. A very good immune serum (hemolytic streptococcus serum 2A) resulted. Its agglutinin titer for hemolytic streptococcus was 1:1,280, for nonhemolytic streptococcus, 1:160; the opsonic index to hemolytic streptococcus was 4, and to nonhemolytic streptococcus, 1; it gave + + + + complement fixation with hemolytic streptococcus antigen, and only + with nonhemolytic streptococcus antigen. The immune serum of the second nonhemolytic streptococcus rabbit (nonhemolytic streptococcus serum 2) was as follows: Agglutination with the homologous suspension was 1:2,560, with the heterologous suspension, 1:10; the opsonic index to the nonhemolytic streptococcus was 2, and to the hemolytic streptococcus, 4; the complement-fixation with the homologous antigen was + + + +, and with the heterologous antigen +. The second nonhemolytic streptococcus

rabbit was also given a resting period of 3 months. There was a considerable drop in antibody content when this time had elapsed. The antibody content, after further immunization (nonhemolytic streptococcus serum 2A) was quite similar to that following the first immunization. Agglutination tests were less specific, since it now agglutinated hemolytic streptococcus bacteria in 1:160; the opsonic indexes were the same; the complement-fixation tests with the homologous antigen gave + + instead of + + + + fixation.

In 1921, two more rabbits were immunized with the strains (hemolytic streptococcus serum 3 and nonhemolytic streptococcus serum 3). The results were similar to those of the previous years, but the antibody content of both serums was so low that they were further immunized (hemolytic streptococcus serum 3A and nonhemolytic streptococcus serum 3A). After 3 weeks the serums became absolutely nonspecific. The agglutinin titer for all was 1:1,280. When the serums were used in the usual amounts all were anticomplementary in the complement-fixation tests, but when the serums were highly diluted, all gave + + + + fixation. Blood was taken several times at 2-day intervals from these rabbits, and the tests were repeated with the same results.

In the summer of 1921, the immune serums, which had been used in the tabulated tests and had been stored in the icebox, were again inactivated, and the tests were all repeated at the same time. The results were almost identical with those obtained from the former tests, an observation also made by Dochez, Avery, and Lancefield.[5]

There was no change in the morphology of the two streptococci during the two years in which they were under observation. At the end of the two years, the hemolytic streptococcus still produced hemolytic colonies on the blood-agar plates, and the nonhemolytic streptococcus still produced green colonies. This is contrary to the observations of Kuszynski and Wolf,[7] that freshly isolated nonhemolytic streptococcus cultivated on blood-agar plates yields hemolytic colonies in every generation until finally the nonhemolytic colonies are of such low vitality that they die out readily. The constancy of the hemolytic variety, noted by Dochez, Avery and Lancefield,[5] Kuszynski and Wolf,[7] Clawson [8] and others, has been widely accepted, although Rosenow [9] was able to transform a number of hemolytic strains into the viridans variety and to bring about the reverse change in strains which were nonhemolytic on isolation. This question has recently

[7] Ztschr. f. Hyg. u. Infektionskr., 1921, 92, p. 119.
[8] Jour. Infect. Dis., 1920, 26, p. 93.
[9] Ibid., 1914, 14, p. 1.

been investigated by Schnitzer and Munter,[10] who claim to have seen the development of green colonies from hemolytic strains in cultures and to have been able to cause such a change much more readily in the animal body. They interpret the loss of the hemolytic property as an evidence of decrease in virulence, those individuals of an inoculated suspension which are least resistant to the protective mechanisms of the host growing out as green, nonhemolytic colonies when subcultivated. In the hemolytic streptococcus from the heart valve, the development of green colonies was never observed, either in repeated platings or in the mouse inoculations which proved the rapid loss of virulence. The sugar fermentations of each strain remained constant throughout the two years.

Serologically, the two strains could be differentiated fairly well by the agglutination and complement-fixation tests. The opsonic tests, contrary to the results of Tunnicliff,[11] who worked with immune hemolytic streptococcus sheep serum, were less specific, since 3 of the 4 hemolytic streptococcus antiserums had higher opsonic indexes to the nonhemolytic streptococcus suspension than to the hemolytic streptococcus bacteria, and only 1 of the nonhemolytic streptococcus antiserums had a higher index to nonhemolytic streptococcus than to hemolytic streptococcus.

The cultural, agglutination and complement-fixation reactions indicate that the two organisms, supposedly distinct before their entrance into the body, maintained their original differences after a period of parasitism in the same host and did not themselves react to the host by biologic changes which might have resulted in similarity of reactions. The two strains could not be differentiated by their opsonin reactions. Whether this means that in this particular instance the phagocytic reaction is a less delicate one than the others, or that, as a result of growth in the same host, mammalian leukocytes are equally stimulated by antiserums against the two organisms, it is impossible to decide. Taking into consideration the serologic tests made with the immune serums from the two strains, the hemolytic streptococcus is a more specific and fixed strain, a conclusion agreeing with the observations of Clawson,[8] Dochez, Avery and Lancefield,[5] Howell [4] and others. If the splitting off of hemolytic variants from Streptococcus viridans occurs, as claimed by Kuszynski and Wolf,[7] one would expect it to occur in the nonhemolytic organism studied, since this strain might originally

[10] Ztschr. f. Hyg. u. Infektionskr., 1921, 93, p. 96.
[11] Jour. Am. Med. Assn., 1920, 75, p. 1339.

have been a variant of the hemolytic strain and might in later cultivation have given rise to hemolytic descendents. No hemolytic colonies ever developed from the viridans strain on frequent replatings during the course of 2 years. The reverse change, the development of nonhemolytic colonies from the hemolytic strain as reported by Schnitzer and Munter,[10] was also not observed.

The hemolytic streptococcus and the nonhemolytic streptococcus isolated from the same diseased heart valve differed in virulence when first isolated. At that time, and persistently for 2 years, they have differed in a constant manner morphologically, culturally, and to a lesser degree serologically. The virulence of each strain was rapidly lost under artificial cultivation.

SUMMARY

From the same aortic lesion in a case of chronic vegetative valvular endocarditis, two strains of streptococci were isolated.

One was a typical Streptococcus hemolyticus, the other a typical Streptococcus viridans. On the basis of sugar reactions the hemolytic organism was Streptococcus pyogenes, the nonhemolytic strain Streptococcus salivarius.

The two strains differed not only in their reactions on blood and sugar mediums, but also in their serologic reactions. A parasitic existence in the same lesion had not acted in such a manner as to tend toward similarity of biologic properties.

The characters of each organism and the differences between the two have remained fixed and constant through a period of 2 years of artificial cultivation.

When first isolated the hemolytic streptococcus was much more highly virulent than the other strain and it had the unusual property of agglutinating rather than hemolyzing red blood corpuscles when grown in fluid mediums. The latter property and the virulence were both lost under cultivation.

The viridans strain studied over a period of 2 years offers no support to the view of Kuszynski and Wolf that Streptococcus viridans gives rise to hemolytic descendents in each successive replating, or to the view of Schnitzer and Munter that viridans colonies may develop from the hemolytic variety as the virulence of the latter decreases.